Germline mutation rate of the microsatellite *HrU*10 in tree swallows, *Tachycineta bicolor*, in an area with high PAH pollution

Master of Science thesis in Evolution and Biodiversity

by

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Preface

Denne masteroppgaven er gjennomført ved Naturhistorisk museum, seksjon for forskning og samlinger, ved Universitetet i Oslo, i perioden august 2007 til juni 2009. Feltarbeidet ble utført i Hamilton, Canada, våren 2008 på et av Environment Canadas feltområder. Laboratoriearbeidet er utført på Naturhistorisk museums DNA-lab av undertegnede.

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Abstract

Previous studies have revealed increased levels of germline mutations in minisatellite DNA in populations of herring gulls and mice sampled in Hamilton Harbour, Ontario Canada, an industrial area with high emissions of genotoxic contaminants like polycyclic aromatic hydrocarbons (PAH). In this study we investigated the occurrence of germline mutations of the highly mutable pentanucleotide microsatellite *HrU*10 in a population of free-living, migratory tree swallows breeding in the same area. We found a mutation rate of 5.3% (17/320 meiotic events) which was not statistically higher than that of a control population breeding in a rural and presumably unpolluted habitat in Eastern Ontario (3.4%; 14/412 meiotic events). In accordance with previous studies of microsatellite mutations, mutations were typical of slippage mutations with loss or gain of one (two) core repeat units, and the likelihood of mutation increased with the size of the microsatellite allele. We can not conclude that there is an effect of PAH on the *HrU*10 mutation rate, for that we would require a considerably larger data set to obtain statistical significance.

Introduction

It is documented that a lot of the PAHs are having mutagenic, carcinogenic and/or teratogenic effects on living organisms (Bleeker et al. 1999; Hall & Oris 1991; Srogi 2007). PAHs occur in oil, tare and coal and are released into the surrounding areas as a by-product of fossil burning (Rogge et al. 1993; Srogi 2007). Another source is steel production and steel recycling (Katz & Chan 1980; Katz et al. 1978; Srogi 2007). PAHs are lipophilic and can therefore easily enter the cells of living tissues where it because of its physical and chemical properties can do mutagenic damage to the DNA (Srogi 2007).

In Hamilton harbour, Ontario, Canada, there is a large industrial site which releases large amounts of PAHs and other pollutants (heavy metals and polychlorinated biphenyls (PCB's)) into the local area (harbour sediment and air) (Legzdins et al. 1993; Marvin et al. 1993) Among the industries there are two steel mills (US Steel Canada and Dofasco Inc.), a metal recycling facility (including lead, iron and aluminium, Poscor Mill Services Corp., which is now owned by 3M Metal), a wastewater treatment plant and a ship maintenance yard. In addition to this is one of the large highways in the area (QEW) passes next to the area. In 1985, the International Joint Commission (IJC) officially recognized Hamilton Harbour as one of the most degraded bodies of water in the Great Lakes (Hall 2006). In this area, studies have been conducted on germline mutation rate of minisatellite DNA in herring gulls, *Larus argentatus* (Yauk et al. 2000; Yauk & Quinn 1996) and laboratory mice (Somers et al. 2002). These studies showed a significant elevation in the germline mutation rate of minisatellites compared to the rural control site.

A problem with minisatellite analyses is that there are multiple gel bands that cannot be attributed to particular loci, and hence the particular mutation events are difficult to study. Microsatellites on the other hand are locus specific and mutations can more easily be tracked back to a particular parent. In a study in Chernobyl on barn swallows an elevated rate of germline mutations in microsatellites was found (Ellegren et al. 1997). Microsatellites are, like minisatellites, a tandemly repeated DNA sequence with no protein coding functions. As far as we know, satellite DNA do not code for anything and is therefore not under any selective pressure (Brown 2006; Ellegren 2004). Microsatellites are variable in length. This is a result of the relative high risk that slippage might occur during DNA replication. Most of the time one or more repeats are either deleted or inserted. And since sexually reproducing organisms inherit one allele from each parent, this makes them good for measuring parentage (Brown 2006) and locus specific germline mutation rates (Anmarkrud et al. 2008; Brohede et al. 2002).

The microsatellite *HrU*10 is a hyper mutable microsatellite that was originally described in the barn swallow, *Hirundo rustica* (Primmer et al. 1996b), but can also be amplified in several other swallow species, including the tree swallow, *Tachycineta bicolor* (Anmarkrud et al. 2008). Due to its hyperpolymorphism, it is a powerful marker in parentage studies (Brohede et al. 2002), but mutations are also a potential source of error. In a rural population of tree swallows near Lake Opinicon, Eastern Ontario, the germline mutation rate of *HrU*10 was 3% (Anmarkrud et al. 2008). It is also a general pattern that the mutation rate of a microsatellite increases with allele size (Anmarkrud et al. 2008; Brohede et al. 2002; Primmer et al. 1996a). Anmarkrud et al. (2008) found that the mutation rate increased from approximately 2% for allele sizes of 200 to 300 bp, to approximately 7% for allele sizes of 500 to 600 bp. Further it seems that most microsatellites have a male-biased mutation rate due to an excess of male

germ cell divisions (Ellegren & Fridolfsson 1997), but this does not seem to be the case with *HrU*10 (Brohede et al. 2002).

In this study, we investigated if tree swallows breeding in the PAH polluted area of Hamilton Harbour have an elevated rate of germline mutations at the *HrU*10 microsatellite as compared to the mutation rates observed in the rural population in Eastern Ontario by Anmarkrud et al. (2008). We would expect the mutation rate to be higher in Hamilton than in the rural habitat in Eastern Ontario, like it is shown in the previous studies from this area (50% to 100% increase in mutation rate) (Somers et al. 2002; Yauk et al. 2000). If so, we would also predict that the increase would be due to a higher mutation rate in the male germ line, due to the fact that sperm cells are produced when birds are on their breeding grounds, and spermatogenesis seems highly sensitive to mutagenic effects (Møller et al. 2008).

Material and methods

Study area

The field work was carried out at Windermere basin, Hamilton (43° 15' 56" N, 79° 46' 51" W), Ontario, Canada (Figure 1), from 18 May to 16 June 2008. The area is in close proximity (0.1-6 km) of a large industrial area, containing two steel mills (Dofasco Inc. is located in industrial sector K and L, US Steel Canada in industrial sector M and N; see Figure 1), a steel recycling (Poscor Mill Services in industrial sector J; see Figure 1), and a big highway, Queen Elizabeth Way (QEW; see Figure 1). In addition, a sewage treatment plant (industrial sector H; see Figure 1) and a ship maintenance yard is situated close by. The land that the nest boxes are on consists of dredge from the lake with about a foot of fill on top. PAHs, PCBs and heavy metals are in the dredge (Marvin et al. 1993; Zeman & Patterson 2003), and the water table is around 2 feet or less below the surface.

In this area there are two nest box plots, Windermere basin south (WBS) and Windermere basin north (WBN). WBS has 35 nest boxes arranged in a grid system and WBN has 34 nest boxes arranged in two rows (see Figure 1). All boxes are facing south east opposite the prevailing winds. The distance between the boxes are approx 2 to 5 meters apart at the side and 1 to 3 meters front to back. In total, 68 of 69 nest boxes were occupied by tree swallows.

Study species

The tree swallow is an insectivorous, migratory passerine bird that breeds in North America and winters in Mexico, Central America and the Caribbean. It is a hole-nester and is easily attracted to nest boxes.

The tree swallow is 13.5 cm long and weighs about 20g. The adult tree swallow has iridescent/metallic blue-green upperparts, white under parts, and a slightly forked tail. The males and females are almost identical in coloration, but the females often have slightly duller colours than the males. Female yearlings (second calendar year) have dull grey-brown plumage, and may thus be separated from older (after second calendar year) females. Males can not be aged on the basis of plumage. Clutch size varies between 4 and 7 eggs, and since only the females incubate the eggs, the sexes can easily be distinguished by the presence or absence of a brood patch. During the breeding season, males can also be recognized on a prominent cloacal protuberance. Females lay one egg per day and starts incubating them when the last or penultimate egg is laid, resulting in relatively synchronous hatching of the clutch. The eggs hatch after 14 days of incubation, and the young leave the nest at approximately day 19 post-hatch (Robertson et al. 1992).

Tree swallows are socially monogamous and territorial, but they have a high degree of promiscuity (Lifjeld et al. 1993) and it was therefore necessary to do a paternity test on all the chicks to identify the true parents.

Field methods

Adults were captured inside their nest box to ensure correct assignment of social parents to their offspring. Males were caught before or during the incubation period, when they entered the box with feathers for nest insulation, or while feeding nestlings. Females were caught during incubation. The first brood hatched 20 May and we begun sampling the hatchlings 1 June. All hatchlings were sampled between day 4 and 12 after hatching. All adults were banded with a US Fish and Wildlife Service aluminium bands issued by the Canadian Wildlife Service. Approximately 10µl blood was collected from each individual by brachial

venipuncture and stored it in Queen's lysis buffer (Seutin et al. 1991) for later genetic analysis. The sampling was done in situ, and all individuals were released immediately after. During our field work period, we observed no death or injuries on any bird as a result of the sampling procedures.

The data set for the parentage study included blood samples of 262 nestlings from 60 broods with both social parents (i.e. 59 fathers and 60 mothers, one male was unpaired and two males were socially polygynous).

Genotyping

DNA was extracted from the blood samples using, e.Z.N.A.™ E-Z® 96 Blood DNA Kit (Omega Bio-Tek, Inc.), protocol 1: E-Z 96® Blood DNA protocol with minor modifications.

Six microsatellites (*Aar*4, *HrU*6, *Ltr*6, *Pdoμ*5, *Tbi*104 and *HrU*10) previously shown to be polymorphic in tree swallows (Delmore et al 2008) were selected for this study. Five of the microsatellites (*Aar*4, *HrU*6, *Ltr*6, *Pdoμ*5 and *Tbi*104) were used to identify both biological parents. In cases where we successfully identified both biological parents we analysed the microsatellite *HrU*10 for mutations. *HrU*10 is a hypermutable microsatellite with a 5 base pair repeat (5'-TTCTC-3') (Anmarkrud et al. 2008; Brohede et al. 2004; Brohede et al. 2002). The allele size of *HrU*10 varies from approximately 200 to 600 base pairs (bp) (Table 1).

The six microsatellite markers (*Aar*4, *HrU*6, *HrU*10, *Ltr*6, *Tbi*104 and *Pdoμ*5) were amplified by using QIAGEN® Multiplex PCR Handbook 02/2008 (QIAGEN) with some modifications. The microsatellite markers were divided into two multiplex PCR combinations. One consisting of *Aar*4, *HrU*6 and *HrU*10, and the other consisting of *Ltr*6, *Tbi*104 and *Pdoμ*5 (all

forward primers were fluorescently dyed). We used 2 µl template DNA and 1.67µM of each primer in a total PCR volume of 10µl. The PCR amplification was carried out on a GeneAmp® PCR System 9700 (Applied Biosystems). The PCR profile was identical for the two multiplexed sets of markers and consisted of an initial activation step at 95°C for 15 min, followed by 30 cycles of a 3-step cycling containing denaturation at 94°C for 30 s, annealing at 57°C for 90 s and extension at 72°C for 60 s, finished with a final extension at 60°C for 30 min. PCR products were run on a 3100 Genetic Analyzer (Applied Biosystems) with GeneScan™-500 ROX™ (Applied Biosystems) as size standard and allele sizes were analysed with GeneMapper v4.0 (Applied Biosystems).

All DNA samples (total of 381, including 262 nestlings) were used in the parentage analysis using five microsatellites (*Aar*4, *HrU*6, *Ltr*6, *Tbi*104 and *Pdoµ*5). This marker set provided a combined probability of non-exclusion of p = 0.0179 with no parents known, and p = 0.0021 with one parent known (Table 1). When assigning parents we allowed no mismatches at any locus, except at *HrU*6 for which a few slippage mutations would be expected to occur (mutation rate of 0.57 %; (Brohede et al. 2002)). In two offspring there was a genotype mismatch at HrU6 consistent with slippage mutation (i.e. one repeat unit) and a complete genotype match with all other markers (including *HrU*10). Hence, parentage was assigned to the putative parent in both cases.

For those nestlings where we identified both genetic parents (i.e. 212 nestlings, see Results) we screened the *HrU*10 alleles for mutation events. With the assumption that the two offspring alleles are inherited with one from each parent we attributed each allelic mismatch to one of the two parents (i.e. the one with no matching alleles) and assumed that the mutated allele was the one most similar in size (Figure 2). If one or both parents or the offspring were

homozygous on the *HrU*10 allele they were discarded from the calculation of mutation rate, due to the risk of null alleles. A null allele is an allele that for some reason does not amplify in the PCR reaction, usually due to a mutation in the priming site, and the individual will erroneously look homozygous for the other allele (DeWoody et al. 2006). The inheritance of a null allele can be confirmed in parentage analysis (parent and offspring apparently homozygous for different alleles) but there is no way of telling whether a mutation has happened. Therefore, for the calculation of mutation rate, we excluded all nestlings that inherited a null allele. We accepted null allele/homozygote's in one or both the parents if the offspring inherited the expressed allele. All mutation data from Anmarkrud et al. (2008) have been recalculated by the same procedure as this, where we have excluded all offspring with null alleles (former mutation rate = 3%, recalculated mutation rate = 3.4%). To verify all mutations we did one rerun of all the mutated offspring and their parents. All allele sizes where confirmed.

Statistical procedures

Statistical analyses were performed using STATISTICA v.7.1 (StatSoft, Inc) Graphs were made by using Origin 7 (OriginLab corp.).

Results

Parentage

Among the 262 offspring, a total of 139 had a complete genotype match with both social parents and were therefore considered as withinpair offspring. The remaining 123 offspring matched with the social mother on all five microsatellite markers but had one or more mismatch with the social father. We considered them as the result of extrapair fertilizations. For a total of 73 of the 123 extrapair offspring, we identified another male in the population with a complete genotype match. Hence we were able to identify both genetic parents for a total of 212 offspring in our data set; 139 withinpair offspring and 73 extrapair offspring. Twenty-eight of the breeding 59 males were identified as an extrapair sire.

Of the 60 broods, 11 consisted of only within pair offspring, 40 had both withinpair and extrapair offspring, and 10 consisted of only extra pair offspring. Hence, 81.7% (49/60) of broods contained one or more extrapair offspring, and 46.6% (122/262) of all offspring were sired extrapair.

Mutation rate

Of the 212 offspring with both parents known, 52 were discarded from this study because of the presence of null-alleles (50 in chicks and 2 in extrapair sire). In the remaining 160 offspring we located 17 germline mutations. This corresponds to a mutation rate of 5.3% (17/320 meiotic events). In all cases the mutated allele deviated by only five or ten base pairs (compared with the most similar sized parental allele) consistent with an insertion or deletion of one or two repeat units from the original allele. Of the 17 mutations we found that, 6 were inherited from the father (4 insertions and 2 deletions) and 11 were inherited from the mother

(3 insertions and 8 deletions whereas one deleted two repeats). In our control population at the rural site the mutation rate was 3.4% (14/412 meiotic events).

The mutation rate in Hamilton was not statistically different from the rural control population (Generalized linear model with binomial error distribution and logit link; $\chi^2 = 1.60$, $p = 0.21$). When we included allele size in the model we found a significant effect of allele size ($\chi^2 = 17.17$, $p < 0.001$) (Figure 3), whereas site remained non-significant ($\chi^2 = 1.22$, $p = 0.269$).

In Hamilton the average allele size of *HrU*10 in adults was 308 bp (n = 190, SD = 76.78) and 305 bp (n = 320, SD = 75.54) in offspring. For adults at our rural control site it was 301 bp (n = 213, SD = 80.81) and 298 bp (n = 412, SD = 73.97). We found significant differences in average allele size between the two sites in both adults and offspring (Mann-Whitney U Test: adults: $Z = 2.12$, $p = 0.034$; offspring: $Z = 2.09$, $p = 0.036$).

Discussion and conclusions

The observed mutation rate was 5.3% in Hamilton and 3.4% in the control area, which indicates a change of 56% in the mutation rate in the predicted direction. However, the difference was not statistically significant and we are therefore unable to reject the null hypothesis of no difference in mutation rate between the areas. The results might well illustrate a problem of low statistical power. Given a 56% increase in mutation rate, a post hoc power analysis (alfa level = 0.05 and power goal = 0.9) show that it would not be possible, with the current sample size in the control area, to gain statistical significant difference in mutation rate, no matter the sample size in Hamilton. However, with a fixed mutation rate of 3.4% (rural mutation rate), we would obtain statistical significance with a sample size of approximately 400 meiotic events. To obtain significance with our present sample size, for both Hamilton (n = 320) and our rural control site (n = 412), a minimum of 22 mutations (6.9% mutation rate) in Hamilton would be required, which corresponds to a 102% increase in mutation rate. In comparison, the previous studies of germline mutation rate in minisatellites in Hamilton found a 100% increase in mutation rate for herring gulls (Yauk et al. 2000) and a 50% to 100% increase in mutation rate for mice (Somers et al. 2002). Both these studies had a considerably larger sample size than our study. When we compare our results with the study on herring gulls (Yauk et al. 2000), we should also take into consideration that herring gulls are nonmigratory and spend all year at the site, while tree swallows are migratory and only in the area during the breeding season. This will probably reduce the amount of mutagens accumulated in tree swallows compared with herring gulls.

Our study confirms previous patterns that mutations are consistent with slippage mutations (Anmarkrud et al. 2008; Ellegren 2004; Wierdl et al. 1997) and increase with allele size. A likely explanation for this is that the longer an allele gets, the higher probability there is for a

misaligned confirmation, and/or for the mismatch-repair system to oversee the misaligned confirmation, which results in a mutation (Anmarkrud et al. 2008; Brohede et al. 2004; Ellegren 2004; Primmer et al. 1996a; Wierdl et al. 1997). We also found that the mutations more frequently had a maternal than a paternal origin, in agreement with previous studies (Anmarkrud et al. 2008; Brohede et al. 2002).

Our study also illustrates the challenges of conducting a mutation study in a species/population with high frequencies of extrapair mating and a high number of candidate fathers. 19% of all chicks had a biological father that was apparently not present in the nest box population. Still we were able to identify the sire of 59% of all extrapair offspring, which is a fairly high fraction compared to other studies of tree swallows (40% of sires at our rural populations (O. Kleven, pers. com.)). The frequency of extrapair paternity was almost identical to the rural control population in Eastern Ontario, which suggest that the high frequency of extrapair paternity does not vary much among tree swallow populations, 46.6% in Hamilton (this study) and 47.9% at our rural population (Delmore et al. 2008). Likewise the proportion of broods containing one or more extrapair offspring was 81.7% in Hamilton (this study) and 82.1% in the rural population (Delmore et al. 2008). There are also previous studies on tree swallows showing similar results (Barber et al. 1996; Dunn et al. 1994; Lifjeld et al. 1993).

In conclusion, we do not have significant results to claim that genotoxic pollution (mainly PAH) heightens the germline mutation rate on the microsatellite *HrU*10. But seen in the light of our small dataset our results might be taken as preliminary evidence and the study should be repeated with a larger data set both for test site and control site.

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Tables and Figures

Table 1
Polymorphism among 6 microsatellite loci, 5 used for parentage analysis and one (*HrU*10) used for mutation measurements in tree swallows (*Trachycineta bicolour*)

Locus	Range	k	N	HObs	Hexp	PIC	NE-1P	NE-2P	HW	F(Null)
*Aar*4	110-142	17	120	0.892	0.880	0.865	0.393	0.244	NS	-0.0084
*Hru*6	150-575	86	120	0.950	0.944	0.938	0.204	0.116	ND	-0.0048
*Ltr*6	179-201	11	120	0.708	0.774	0.743	0.601	0.421	NS	+0.0469
*Tbi*104	230-255	13	120	0.750	0.724	0.704	0.644	0.453	NS	-0.0208
*Pdoμ*5	215-231	8	120	0.758	0.793	0.763	0.576	0.396	NS	+0.0154
*Hru*10	189-592	117	119	0.824	0.991	0.987	0.051	0.026	ND	+0.0905

Data on the number of alleles (k), number of genotyped adult individuals (N), observed heterozygosity (HO), expected heterozygosity (HE), mean polymorphic information content (PIC), probability of exclusion assuming no parent known (NE-1P), probability of exclusion assuming one parent known (NE-2P), Hardy-Weinberg (HW) (not significant-NS or no data-ND), and estimated frequency of null alleles (F(Null)) are presented.

Figure 1: Satellite picture (from Google™ Earth) of placement of nest box sites WBN and WBS in relation to industrial area (red dots symbolize nest box placement).

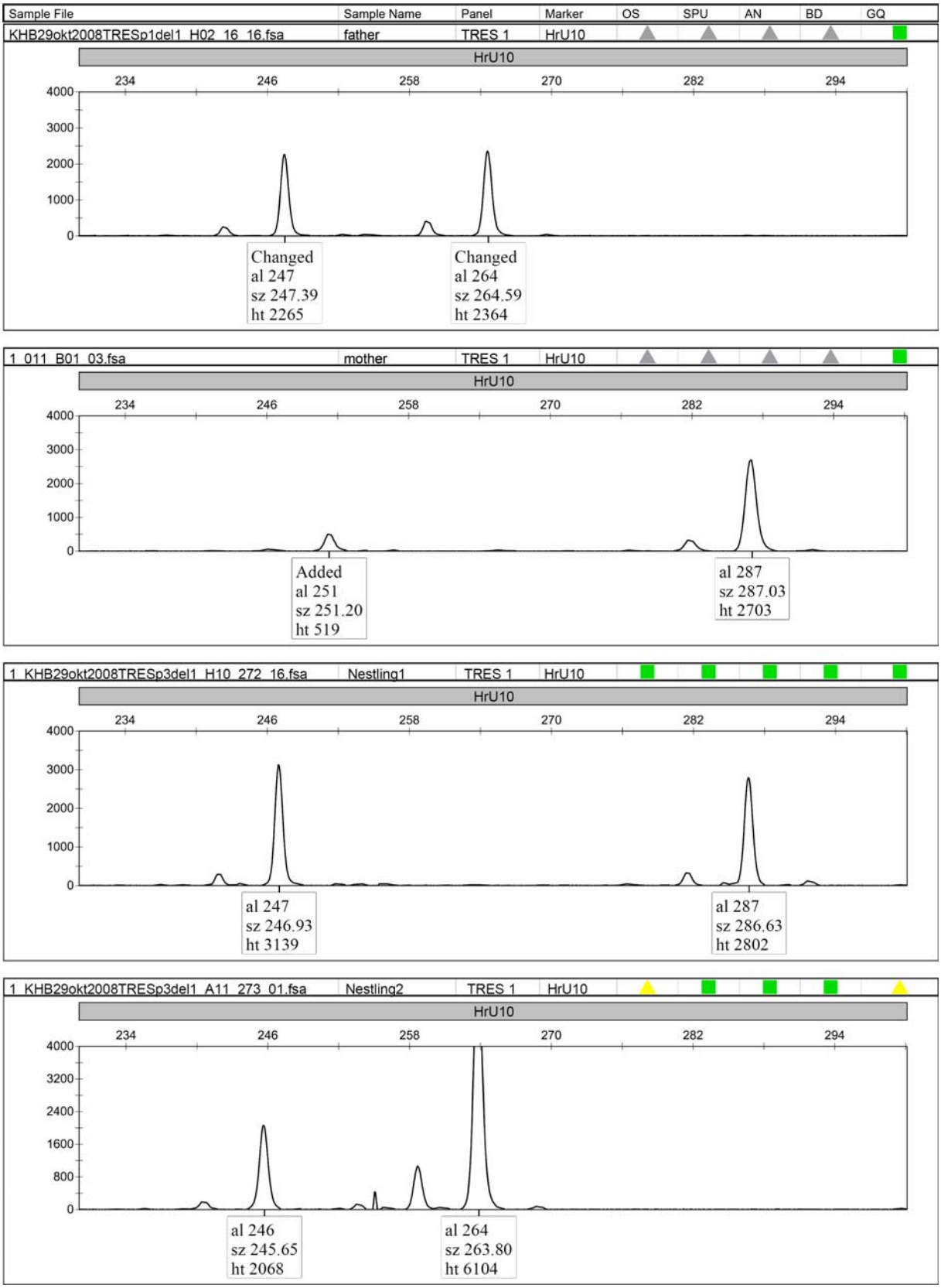

Figure 2. Electropherograms of the pentanucleotide microsatellite HrU10, showing a germline mutation event of the 251bp mother allele to the 246bp (5bp or one repeat deletion) allele in nestling2.

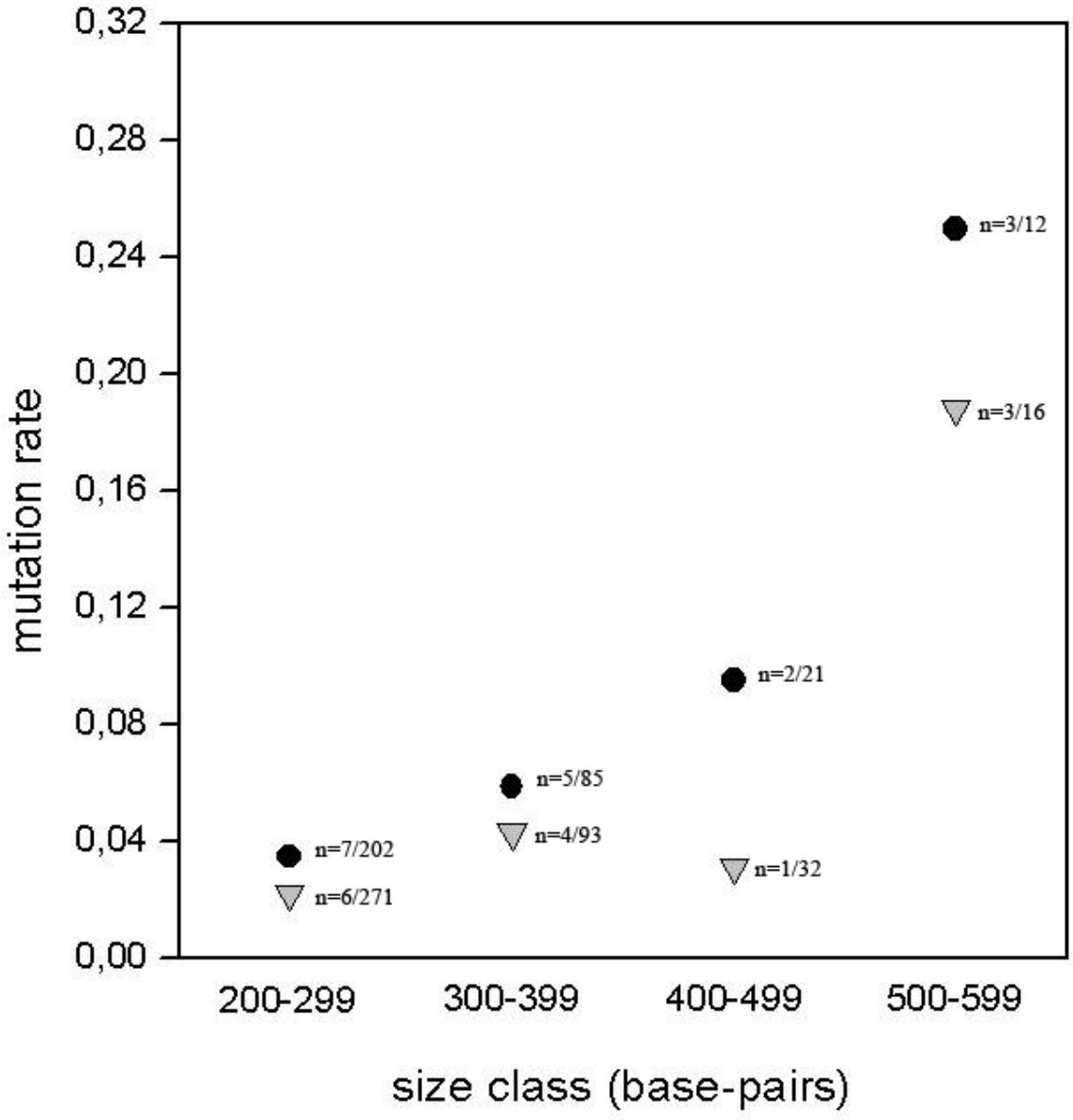

Figure 3. Relationship between mutation rate and allele size, where black circles = Hamilton and gray triangles = control. The alleles are lumped into 4 size categories: 200-300, 300-400, 400-500 and 500-600. n = (mutated alleles) / (total number of meiotic events) within each allele size category.